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(54) **Tests based on nucleic acids of endosymbiont cellular organelles**

(57) The invention relates to the diagnosis of disease or the determination of functioning of cellular organisms, being of multi-cellular or unicellular nature, being visible by the naked eye or being a micro-organism. The invention provides a method for determining func-

tioning of a cellular organism comprising determining the relative ratio of a first endosymbiont cellular organelle nucleic acid and/or gene product thereof in a sample obtained from said organism.

EP 1 211 323 A1

Description

[0001] The invention relates to the diagnosis of disease or the determination of functioning of cellular organisms, being of multi-cellular or unicellular nature, being visible by the naked eye or being a micro-organism.

[0002] A diagnostician of disease is a student of (mal)functioning of cellular organisms. Said student classically can employ a broad range of inroads into said organism to obtain relevant information as to the various aspects of said malfunctioning. These inroads vary widely, examples range from detecting relative ratios of kidney stones by studying urinary samples obtained from various patients, from probing for the presence or absence of intestinal ulcers via endoscopy, from scanning for detectable tumors by nuclear magnetic resonance (NMR), from detecting diabetes by testing for insulin levels and/or glucose concentration in blood plasma, from determining cancer proneness by determining transcriptional levels of oncogenes, and so on.

[0003] Currently, detection of disease or malfunctioning (or vice versa, of health and proper functioning) of higher organisms, such as animals and plants relies very much on testing samples obtained from these organisms and studying these samples in a laboratory. Often when a fruitful method capable of determining, identifying or detecting (aspects of) a disease or malfunctioning of an organism has been found it is in general also useful in testing or screening for compounds or methods useful for treatment of (aspects of) said disease or malfunctioning or useful in testing or screening for compounds or methods involved in causing (aspects of) said disease or malfunctioning; by using the same or a similar method as used in diagnosis it is generally possible to generate an assessment of the usefulness of such candidate compounds or methods in treating and/or causing the disease or malfunctioning in question. Clearly, life science laboratories are always in the need of yet other inroads into organisms to obtain yet more information relating to disease or malfunctioning and to compounds and methods related to cause and/or treatment of disease or malfunctioning.

[0004] The invention provides a method for determining (mal)functioning of an cellular organism comprising determining the relative ratio of an endosymbiont cellular organelle nucleic acid and/or or gene product thereof in relation to another nucleic acid or gene product present in a sample obtained from said organism. Endosymbiont cellular organelles are those organelles of an eukaryotic cell that are thought to have been derived of prokaryotic bacteria very early on in the evolution of eukaryotic cells; these bacteria (is thought) have engaged in an symbiosis with early eukaryotic cells, and at present, eukaryotic cells comprising these endosymbiont organelles in general cannot live without them; none of the present eukaryotic cells would function properly without mitochondria, and most plant cells would at least considered to be malfunctioning when no proplastids, or organelles derived thereof, such as chloroplasts, etioplasts, amyloplasts, elaioplasts or chromoplasts were present. These organelles in general appear to be at least partially self-replicating bodies which, although under some nuclear controls, still possess considerable autonomy.

[0005] In particular, the invention provides a method whereby said relative ratio of an endosymbiont cellular organelle nucleic acid and/or or gene product thereof is determined in relation to the amount of essentially nuclear nucleic acid detectable in said sample (be it DNA or RNA), or in relation to gene products (derivable by transcription and/or translation, such as mRNA or (poly)peptides) of said nuclear nucleic acid, (nuclear nucleic acid herein comprises chromosomal DNA and the RNA transcribed therefrom) for example present in nuclear or cytoplasmatic fractions or parts of said sample. DNA or corresponding mRNA encoding components of small nuclear ribonucleoprotein (SNRNP), or other essentially common nucleic acid derived from chromosomal DNA, is particularly useful to test, because of its ubiquitous presence. In this way, the invention provides a method for studying for example mitochondrial and/or proplastid related disease. Endosymbiont cellular organelle related disease encoded by defects in said organelle's DNA manifests in many different syndromes and is often variable in its expression (and thus in general hard to detect by testing for clinical parameters alone) due to heteroplasmy, whereby mutant and wild type nucleic acid can be found in one cell, whereby its distribution can vary. Endosymbiont cellular organelle related disease is often aggravated with increasing age of the affected individual. Endosymbiont cellular organelle related disease can also often be observed after treatment against other disease with various drugs, and than contributes to various side-effects of those drugs that basically one would like to avoid during treatment. Those side effects can now be better studied by using a method as provided herein.

[0006] Furthermore, the invention provides a method whereby said relative ratio of an endosymbiont cellular organelle nucleic acid and/or or gene product thereof is determined in relation to the amount of a distinct endosymbiont cellular organelle nucleic acid detectable in said sample (be it DNA or RNA), or in relation to gene products (derivable by transcription and/or translation, such as mRNA or (poly)peptides) of said endosymbiont cellular organelle nucleic acid. In one aspect of the invention the method involves determining a ratio between organelle DNA, such as mtDNA, and the corresponding transcriptionally derivable organelle RNA, in the example the related mtRNA, or translated gene product. In yet another aspect of the invention, the ratio between two distinct organelle DNA's or related gene products is determined.

[0007] Furthermore, the invention provides a diagnostic kit comprising at least one means for performing a method according to the invention, said kit comprising at least one primer or probe set selective for the amplification and

detection of a nucleic acid related to or derived from endosymbiont cellular organelles and, when so desired, necessary amplification reagents, such as can be found exemplified in the detailed description herein or otherwise known in the art. In particular, the invention provides a diagnostic kit wherein said kit comprises more than one primer or probe set for the amplification of nucleic acid sequences related to cellular organelles, preferably supplemented with a primer or probe set for the amplification of nucleic acid related to the chromosomes, such as a SNRNP specific primer or probe. In particular the invention provides a kit comprising at least one primer or probe from table 1 for the amplification of nucleic acid sequences related to cellular organelles. It is of course preferred that said amplification reagents, when provided with the kit, comprise an enzyme with reverse transcriptase activity, such as required for PCR or NASBA amplification. Of course, a kit comprising a means for the detection of a geneproduct other than nucleic acid, for use in a method according to the invention is herewith also provided.

[0008] Other possible uses of the invention lay in candidate drug testing, for beneficial activity and/or side effects of possible medicaments or pharmaceutical compositions such as candidate anti-parasitic compounds, antibiotic compounds, cytostatic compounds, and so on. For example, the invention provides a method for determining therapeutic activity and/or possible side-effects of a candidate compound, for example in determining its usefulness for treatment of malfunctioning of a cellular organism, comprising determining the relative ratio of an endosymbiont cellular organelle nucleic acid and/or gene product thereof in a sample obtained from an organism, preferably said organism or an essentially related organism, such as belonging to the same species or genus, having been provided with said compound. Also use for (selective) toxin testing, of e.g. herbicides, insecticides, anti-parasitic compounds, antibiotic compounds is here provided, the invention provides a method for determining toxic activity of a candidate compound, for example in determining its usefulness for causing malfunctioning of a cellular organism, e.g. by having a cytostatic or even cytotoxic effect, comprising determining the relative ratio of an endosymbiont cellular organelle nucleic acid and/or gene product thereof in a sample obtained from an organism, preferably said organism or related organism, having been provided with said compound.

[0009] In a preferred embodiment, selectivity is also tested, using or applying the method as provided herein (preferably in parallel experiments) on or to a first organism and on or to an essentially unrelated second organism, if desired belonging to a different family or order, but preferably belonging to at least a different class or phylum, most preferably belonging to a different kingdom of organisms. Selectivity aspects are for example tested by testing the compounds in (if desired only in cells of) a first target organism (such as a bacterium or parasite) as well as of testing the host or cells thereof, being an essentially unrelated second organism, for example a mammal or plant, or by testing of a crop plant or cells thereof as well as testing an essentially unrelated weed plant or cells thereof with said compound, to determine for example selective toxic or selective therapeutic effects. It is also provided to test normal cells derived from an individual in parallel or comparison with aberrant cells, such as tumour cells derived from the same individual, to detect or screen for a tumour-specific or at least selective cytostatic or cytotoxic compound for use in therapy of said individual or others with similar or related disease.

[0010] The invention furthermore provides the use of a compound obtainable or detectable by a method according to the invention in the preparation of a medicament, a herbicide, insecticide, anti-parasiticum, cytostatic, etc. and a medicament, herbicide, insecticide, anti-parasiticum etc. obtainable or derivable or identifiable by a method according to the invention.

[0011] The invention is further explained in the detailed description herein, wherein most examples are directed by way of example at testing of mitochondriae, being central to the provision and use of energy in a cell, however, it will easily be understood that the same principles apply to tests using other endosymbiont organelles, such as chloroplasts, being central to the provision of carbohydrates to a plant cell.

Figure legends

[0012] Figure 1. Examples of standard curves for DNA and RNA target sequences.

[0013] Figure 2. Ratio of mitochondrial DNA and chromosomal DNA in fibroblast cells cultured in the presence of DDC.

[0014] Figure 3. Ratio of mitochondrial RNA and chromosome encode RNA in fibroblast cells cultured in the presence of DDC.

[0015] Figure 4. Ratio of mitochondrial RNA and chromosomal DNA in fibroblast cells cultured in the presence of DDC.

[0016] Figure 5. Ratio of mitochondrial RNA and mitochondrial DNA in fibroblast cells cultured in the presence of DDC.

[0017] Figure 6. Ratio of chromosome encoded RNA and chromosomal DNA in fibroblast cells cultured in the presence of DDC.

[0018] Figure 7. Ratio of mitochondrial DNA and chromosomal DNA in fibroblast cells cultured in the absence of DDC after being cultured with DDC for 4 weeks.

[0019] Figure 8. Ratio of mitochondrial RNA and chromosome encoded RNA in fibroblast cells cultured in the absence of DDC after being cultured with DDC for 4 weeks.

[0020] Figure 9. Ratio of mitochondrial DNA and chromosomal DNA in PBMC's cultured in the presence of DDC for 5 days.

5 [0021] Figure 10. Ratio of mitochondrial RNA and chromosome encoded RNA in PBMC's cultured in the presence of DDC for 5 days.

[0022] Figure 11. Comparison of SNRNP DNA NASBA reactions with and without pre-treatment with restriction enzyme Msp I.

10 Examples Endosymbiont Cellular Organelle Diagnostics

Used ingredients and general methodology

[0023] In table 1 the primers and probes used in the examples are summarised.

15 Standard NASBA nucleic acid amplification reactions were performed in a 20µl reaction volume and contained: 40mM Tris-pH 8.5, 70mM KCl, 12mM MgCl₂, 5mM dithiothreitol, 1mM dNTP's (each), 2mM rNTP's (each), 0.2µM primer (each), 0.05µM molecular beacon, 375mM sorbitol, 0.105 µg/µl bovine serum albumin, 6.4 units AMV RT, 32 units T7 RNA polymerase, 0.08 units RNase H and input nucleic acid. The complete mixture, except the enzymes, sorbitol and/or bovine serum albumin was, prior to adding the enzyme mixture, heated to 65°C for 2 minutes in order to denature any

20 secondary structure in the RNA and to allow the primers to anneal. After cooling the mixture to 41°C the enzymes were added. The amplification took place at 41°C for 90 min in a fluorimeter (CytoFluor 2000) and the fluorescent signal was measured every minute (using the filter set 530/25 nm and 485/30 nm). For amplification of DNA target sequences the 65°C denaturation step was replaced with a 95°C denaturation step for the same period of time. To achieve quantification, a dilution series of target sequence for a particular primer set was amplified and the time

25 points at which the reactions became positive (the time to positivity, TTP) were plotted against the input amounts of nucleic acid. This way a calibration curve was created that could be used to read TTP values of reactions with unknown amounts of input and deduce the input amount. Examples of typical standard curves for quantification of RNA and DNA are shown in figure 1. For some of the target sequences no dilution series were available with reliable absolute amount of copies determined. Those series were given an arbitrary unit as measurement instead of DNA or RNA

30 copies, e.g. cell-equivalent or ET-unit. As a result it sometimes seems that there is less RNA than DNA, which is quite the opposite of what is expected.

Cells (fibroblasts and PBMC's) were cultured under standard conditions in standard media known to persons skilled in the art with addition of drugs or putative toxic or stimulating compounds as defined in the examples. Nucleic acids were isolated from the cells with the method described by Boom et al (Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim-van Dillen PM, van der Noordaa J, 1990. Rapid and simple method for purification of nucleic acids. *J Clin Microbiol*; 28(3):495-503) or with dedicated isolation kits purchased from Qiagen (Qiagen GmbH, Max Volmer Strasse 4, 40724 Hilden, Germany) and used according to the manufacturer's protocols. A small aliquot of the isolated nucleic acid was analysed on an agarose gel and the remainder stored at -80°C until further analysis. Usually the nucleic acid was diluted 10 times with water and of the diluted nucleic acid usually 5 µl was used as input in the NASBA amplification

40 reactions.

Example 1

[0024] In this example it is explained which ratio's can be measured with a method according to the invention and the meaning they can have in diagnostic sense:

45 The invention for example provides determining the relative ratio of organelle DNA to chromosomal DNA. This ratio, when compared with normal values or determined at at least two points in time, shows the decline or increase of organelles per cell. Also is provided determining the ratio of organelle RNA to chromosome encoded RNA. This ratio when compared with normal values or determined at at least two points in time, shows the organelle transcription

50 activity decline or increase per cell, normalised for the active state (i.e. transcription state) of the cell. Also is provided determining the ratio of organelle RNA to chromosomal DNA. This ratio when compared with normal values or determined at at least two points in time, shows the organelle transcription activity decline or increase per cell. Also is provided determining the ratio of organelle DNA to organelle RNA. This ratio, when compared with normal values or determined at at least two points in time, shows the decline or increase of transcription in the organelle,

55 indicating regulation at the transcriptional level to achieve a certain mRNA (and therefore protein) level. Also is provided determining the ratio of organelle DNA to chromosome encoded RNA. This ratio, when compared with normal values or determined at at least two points in time, shows the decline or increase of transcription in the cell, in relation to chromosomal RNA transcription levels, indicating the activity state of the organelle, which is especially useful

when chromosomal RNA is determined that encodes an organelle protein or other component thereof.

Example 2

5 [0025] Fibroblast cells were cultured *in vitro* in the presence of the anti viral drugs DDC, AZT and D4T at two concentrations each, 3 μ M and 30 μ M, respectively, for 4 weeks. As controls cell cultures with ethidium bromide and without drugs were also performed. Ethidium bromide is known to deplete mitochondrial DNA completely from cells and is a positive control in terms of achieving an effect on the mitochondria content of cells. At one week intervals part of the cells was harvested and analyzed for amount of mitochondrial DNA (primers MtD p1 and MtD p2 and probe MtD mb) and chromosomal DNA (primers SnrpD p1 and SnrpD p2 and probe SnrpD mb) in the described NASBA protocol. The cultures with AZT, D4T and without additive showed no measurable change in mitochondrial DNA to chromosomal DNA ratio in the culture period of 4 weeks. The culture with Ethidium bromide showed a decline in mitochondrial DNA content as expected. The results for DDC are shown in figure 2. The data in figure 2 clearly show a decline in the amount of mitochondrial DNA per cell with more than 2 logs and therewith the mitochondrial toxicity of the antiviral drug DDC.

Example 3

20 [0026] Fibroblast cells were cultured *in vitro* in the presence of the anti viral drugs DDC, AZT and D4T at two concentrations each, 3 μ M and 30 μ M, respectively, for 4 weeks. As controls cell cultures with ethidium bromide and without drugs were also performed. Ethidium bromide is known to deplete mitochondrial DNA completely from cells and is a positive control in terms of achieving an effect on the mitochondria content of cells. At one week intervals part of the cells was harvested and analyzed for amount of mitochondrial RNA (primers MtR p1 and MtR p2 and probe MtR mb) and chromosome encoded RNA (primers SnrpR p1 and SnrpR p2 and probe SnrpR mb) in the described NASBA protocol. The cultures with AZT, D4T and without additive showed no measurable change in mitochondrial RNA to chromosome encoded RNA ratio in the culture period of 4 weeks. The culture with Ethidium bromide showed a decline in mitochondrial RNA content as expected. The results for DDC are shown in figure 3. The data in figure 3 clearly show a decline in the amount of mitochondrial RNA per cell with at least 2 logs and therewith the mitochondrial toxicity of the antiviral drug DDC. The time point at 3 weeks has a very low value and it presumably this is somewhat of a outlier measurement.

Example 4

35 [0027] Fibroblast cells were cultured *in vitro* in the presence of the anti viral drugs DDC, AZT and D4T at two concentrations each, 3 μ M and 30 μ M, respectively, for 4 weeks. As controls cell cultures with ethidium bromide and without drugs were also performed. Ethidium bromide is known to deplete mitochondrial DNA completely from cells and is a positive control in terms of achieving an effect on the mitochondria content of cells. At one week intervals part of the cells was harvested and analyzed for amount of mitochondrial RNA (primers MtR p1 and MtR p2 and probe MtR mb) and chromosomal DNA (primers SnrpD p1 and SnrpD p2 and probe SnrpD mb) in the described NASBA protocol. [0028] The cultures with AZT, D4T and without additive showed no measurable change in mitochondrial RNA to chromosomal DNA ratio in the culture period of 4 weeks. The culture with Ethidium bromide showed a decline in mitochondrial RNA content as expected. The results for DDC are shown in figure 4. [0029] The data in figure 4 clearly show a decline in the amount of mitochondrial RNA per cell with almost 3 logs and therewith the mitochondrial toxicity of the antiviral drug DDC. The time point at 3 weeks has a very low value and it presumably this is somewhat of a outlier measurement.

Example 5

50 [0030] Fibroblast cells were cultured *in vitro* in the presence of the anti viral drugs DDC, AZT and D4T at two concentrations each, 3 μ M and 30 μ M, respectively, for 4 weeks. As controls cell cultures with ethidium bromide and without drugs were also performed. Ethidium bromide is known to deplete mitochondrial DNA completely from cells and is a positive control in terms of achieving an effect on the mitochondria content of cells. At one week intervals part of the cells was harvested and analyzed for amount of mitochondrial RNA (primers MtR p1 and MtR p2 and probe MtR mb) and mitochondrial DNA (primers MtD p1 and MtD p2 and probe MtD mb) in the described NASBA protocol. [0031] The cultures with AZT, D4T and without additive showed no measurable change in mitochondrial RNA to mitochondrial DNA ratio in the culture period of 4 weeks. The culture with Ethidium bromide showed a decline in mitochondrial RNA and DNA content as expected. The results for DDC are shown in figure 5. [0032] The data in figure 5 clearly show that the ratio of mitochondrial DNA to RNA is not significantly changing over

the period of 4 weeks. The time point at 3 weeks in figure 5 has a low value for mitochondrial RNA that shows up, this measurement is presumably somewhat of an outlier measurement.

Example 6

[0033] Fibroblast cells were cultured *in vitro* in the presence of the anti viral drugs DDC, AZT and D4T at two concentrations each, 3 μ M and 30 μ M, respectively, for 4 weeks. As controls cell cultures with ethidium bromide and without drugs were also performed. Ethidium bromide is known to deplete mitochondrial DNA completely from cells and is a positive control in terms of achieving an effect on the mitochondria content of cells. At one-week intervals part of the cells was harvested and analyzed for amount of chromosome encoded RNA (primers SnrpR p1 and SnrpR p2 and probe SnrpR mb) and chromosomal DNA (primers SnrpD p1 and SnrpD p2 and probe SnrpD mb) in the described NASBA protocol.

The cultures with AZT, D4T, ethidium bromide and without additive showed no measurable change in ratio in the culture period of 4 weeks. The results for DDC are shown in figure 6.

The data in figure 6 clearly show that the ratio of chromosomal DNA to RNA is not significantly changing over the period of 4 weeks.

Example 7

[0034] Fibroblast cells were cultured *in vitro* in the presence of the anti viral drug DDC at a concentration of 30 μ M for 4 weeks. After that period the cell culture continued but now in the absence of DDC. During this period of culture without DDC part of the cells was harvested and analyzed for amount of mitochondrial DNA (primers MtD p1 and MtD p2 and probe MtD mb) and chromosomal DNA (primers SnrpD p1 and SnrpD p2 and probe SnrpD mb) in the described NASBA protocol at two-week intervals for a period of 12 weeks. The results of the analysis are shown in figure 7. The results in figure 7 clearly show that the amount of mitochondria per cell increases with more than 2 logs after DDC is removed from the culture. This results shows that the toxic effect of DDC can be reversed if there are still some mitochondria left in the cells to repopulate the new growing cells.

Example 8

[0035] Fibroblast cells were cultured *in vitro* in the presence of the anti viral drug DDC at a concentration of 30 μ M for 4 weeks. After that period the cell culture continued but now in the absence of DDC. During this period of culture without DDC part of the cells was harvested and analyzed for amount of mitochondrial RNA (primers MtR p1 and MtR p2 and probe MtR mb) and chromosome encoded RNA (primers SnrpR p1 and SnrpR p2 and probe SnrpR mb) in the described NASBA protocol at two-week intervals for a period of 12 weeks. The results of the analysis are shown in figure 8.

The results in figure 8 clearly show that the amount of mitochondrial RNA per cell increases with more than 2 logs after DDC is removed from the culture. This results shows that the toxic effect of DDC can be reversed and that the function of the mitochondria comes back as shown by synthesis of RNA and subsequently proteins.

Example 9

[0036] Fresh peripheral blood mononuclear cells (PBMC's) from a healthy blood donor were cultured *in vitro* in the presence of the anti viral drugs DDC, AZT and D4T at two concentrations each, 6 μ M and 60 μ M, respectively, for 5 days. As controls cell cultures with DMSO and without drugs were also performed. DMSO is part of the solvent in which the drugs are solubilized. After 5 days the cells were harvested and analyzed for amount of mitochondrial DNA (primers MtD p1 and MtD p2 and probe MtD mb) and chromosomal DNA (primers SnrpD p1 and SnrpD p2 and probe SnrpD mb) in the described NASBA protocol.

[0037] The cultures with AZT, D4T, DMSO and without additive showed no measurable change in ratio in the culture period 5 days. The results for DDC are shown in figure 9.

The results in figure 9 clearly show the decline in PBMC's of mitochondrial DNA per cell of more than 1 log during the 5 day culture period.

Example 10

[0038] Fresh peripheral blood mononuclear cells (PBMC's) from a healthy blood donor were cultured *in vitro* in the presence of the anti viral drugs DDC, AZT and D4T at two concentrations each, 6 μ M and 60 μ M, respectively, for 5 days. As controls cell cultures with DMSO and without drugs were also performed. DMSO is part of the solvent in which

the drugs are solubelized. After 5 days the cells were harvested and analyzed for amount of mitochondrial RNA (primers MtR p1 and MtR p2 and probe MtR mb) and chromosome encoded RNA (primers SnrpR p1 and SnrpR p2 and probe SnrpR mb) in the described NASBA protocol.

[0039] The cultures with AZT, D4T, DMSO and without additive showed no measurable change in ratio in the culture period 5 days. The results for DDC are shown in figure 10. Interestingly, the results in figure 10 do not clearly show a decline in PBMC's of mitochondrial RNA per cell during the 5-day culture period at the highest concentration of DDC used. This is in contrast to the mitochondrial DNA as shown in example 9. It is hypothesized that the decline in mitochondrial DNA is compensated by an increase in transcription, maintaining the level of mitochondrial RNA. This mechanism delays the decline of mitochondrial RNA.

Consequently, one can say that the mitochondrial RNA is a reflection of the current status of the functionality of the mitochondria and that mitochondrial DNA is predictive of what will happen in the (near) future with the mitochondrial function and therefore has a more prognostic character.

Example 11

[0040] Using the primers and probes Rubisco-DNA p1, Rubisco-DNA p2, Rubisco-DNA MB, Rubisco-RNA p1, Rubisco-RNA p2 and Rubisco-RNA-MB (table 1) the chloroplast DNA and RNA of *Oryza sativum* (rice) can be quantified and the ratio to the chromosomal DNA and RNA can be determined by using primers and probes OryzaDNA p1, OryzaDNA p2, OryzaDNA mb, OryzaRNA p1, OryzaRNA p2, OryzaRNA mb (table 1). During the application of herbicide (or other) compounds the conditions of the plants can be assessed by measurement of the chloroplast nucleic acid content of the cells using amplification methods like PCR and NASBA that are known to persons skilled in the art. At the same time, using primer sets suitable for weeds, the deterioration of the unwanted plants can be monitored. It is clear that these molecular tools are very suited in the research for new herbicides that specifically attack one group of plants and not others.

Example 12

[0041] In this example the NASBA nucleic acid amplification reactions for DNA target sequences were performed in a 20µl reaction volume and contained: 40mM Tris-pH 8.5, 70mM KCl, 12mM MgCl₂, 5mM dithiothreitol, 1mM dNTP's (each), 2mM rNTP's (each), 0.2µM primer (each), 0.05µM molecular beacon, 1.5 units restriction enzyme Msp I, 375mM sorbitol, 0.105 µg/µl bovine serum albumin, 6.4 units AMV RT, 32 units T7 RNA polymerase, 0.08 units RNase H and input nucleic acid. The complete mixture, except the enzymes, sorbitol and bovine serum albumin was, prior to adding the enzyme mixture, incubated at 37°C for 25 minutes and subsequently heated to 95°C for two minutes in order to denature the DNA and to allow the primers to anneal. After cooling the mixture to 41°C the enzyme mixture was added. The amplification took place at 41°C for 90 min in a fluorimeter (CytoFluor 2000) and the fluorescent signal was measured every minute (using the filter set 530/25 nm and 485/30 nm).

[0042] To achieve quantification, a dilution series of target sequence for a particular primer set was amplified and the time points at which the reactions became positive (the time to positivity, TTP) were plotted against the input amounts of nucleic acid. This way a calibration curve was created that could be used to read TTP values of reactions with unknown amounts of input and deduce the input amount. Fresh peripheral blood mononuclear cells (PBMC's) from a healthy blood donor were cultured *in vitro* for 5 days. After 5 days the cells were harvested and analyzed for amount of chromosomal DNA (primers SnrpD p1 and SnrpD2 p2 and probe SnrpD mb) with the described NASBA protocol in the chapter "Used ingredients and general methodology" and compared with the NASBA protocol as described in this example. As can be clearly seen in figure 11 the DNA NASBA reactions with pre-treatment of restriction enzyme perform much better than without. The rationale for this observation is the direct extension from the Msp I created 3' over the T7 promoter part of the p1 primer.

Example 13

[0043] Using the primers and probes tRNA-L-D p1, tRNA-L-D p2, tRNA-L-D MB, petB RNA p1, petB RNA p2 and petB RNA MB (table 1) the chloroplast DNA and RNA of *Oryza sativum* (rice) can be quantified and the ratio to the chromosomal DNA and RNA can be determined by using primers and probes OryzaDNA p1, OryzaDNA p2, OryzaDNA mb, OryzaRNA p1, OryzaRNA p2, OryzaRNA mb (table 1). During the application of herbicide (or other) compounds the conditions of the plants can be assessed by measurement of the chloroplast nucleic acid content of the cells using amplification methods like PCR and NASBA that are known to persons skilled in the art. At the same time, using primer sets suitable for weeds, the deterioration of the unwanted plants can be monitored. It is clear that these molecular tools are very suited in the research for new herbicides that specifically attack one group of plants and not others.

Table 1. Sequences of primers and probes used in the examples.

Name	Sequence ¹
MtD p1	5' <i>AATTCTAATACGACTCACTATAGGG</i> GAGAAGAGCCGTTGAGTTGTGGTA 3'
MtD p2	5' TCTCCATCTATTGATGAGGGTCTTA 3'
MtD mb	5' GCATGCCCCCTCCTAGCCTTACTACTAATGCATGC
SnrpD p1	5' <i>AATTCTAATACGACTCACTATAGGG</i> GAGAGGCCCGCATGTGGTGCATAA 3'
SnrpD p2	5' TTCCTTACATCTCTCACCCGCTA 3'
SnrpD mb	5' GCATGCTGTAACCACGCACTCTCCTCGCATGC 3'
SnrpD2 p2	5' TGCGCCTCTTCTGGGTGTT 3'
MtR p1	5' <i>AATTCTAATACGACTCACTATAGGG</i> GAGAGAAGATGGTTAGGTCTAC 3'
MtR p2	5' CGATATGGCGTTCCCCGCATAA 3'
MtR mb	5' GCTCCG AAGCTTCTGACTCTTACCTCCC CGGAGC 3'
SnrpR p1	5' <i>AATTCTAATACGACTCACTATAGGG</i> AGAGGCCCGCATGTGGTGCATAA 3'
SnrpR p2	5' CAGTATGCCAAGACCGACTCAGA 3'
SnrpR mb	5' CGTACGAGAAGAGGAAGCCCAAGAGCCACGTACG 3'
Rubisco-DNA p1	5' <i>AATTCTAATACGACTCACTATAGGGGG</i> GATAATTTCACTACCTTCACGAG 3'
Rubisco-DNA p2	5' GGAGTCCTGAACTAGCCGCAG 3'
Rubisco-DNA MB	5' GCATGCGGTAGATAAACTAGATAGCTAGGCATGC 3'
Rubisco-RNA p1	5' <i>AATTCTAATACGACTCACTATAGGGG</i> GAGTTGTTGTTATTGTAAGTC 3'
Rubisco-RNA p2	5' CAAGTCTTATGAATTCCTATAG 3'
Rubisco-RNA-MB	5' GCTAGCACACAGGGTGTACCCATTATGCTAGC 3'
OryzaDNA p1	5' <i>AATTCTAATACGACTCACTATAGGGGG</i> GATCTTAATTACATGCCGTCA 3'
OryzaDNA p2	5' AAAGTGCCGGTTCTCACTA 3'
OryzaDNA mb	5' GCTAGCCTCTGCAAGCTTCATCAGTAATAGGCTAGC 3'
OryzaRNA p1	5' <i>AATTCTAATACGACTCACTATAGGGG</i> GCTAATGCCCTTTCTTTCTTCCTC 3'
OryzaRNA p2	5' CATATTGGCT TTCGAAGATT 3'
OryzaRNA mb	5' GCTAGCCTTCAGCCATTATCAAGAT GGTGGCTAGC 3'
tRNA-L-D p1	5' <i>AATTCTAATACGACTCACTATAGGGGG</i> TTCTAGTTCGAGAACCCTTG 3'
tRNA-L-D p2	5' GCGAAATCGGTAGACGCTACG 3'
tRNA-L-D MB	5' GCTAGCCAACCTCCAAATTCAGAGAAGCTAGC 3'
petB RNA p1	5' AATTCTAATACGACTCACTATAGGGAAACCGGTAGCAACTTGTACTAG 3'
petB RNA p2	5' GGTTTCGGTATCTCTGGAATATGAG 3'
petB RNA MB	5' GCTAGCGAGGAACGTCTTGAGATTCAGCTAGC 3'

1. The T7 promoter part of primer p1 sequences is shown in *italics*, the stem sequences of the molecular beacon probes are shown in **bold**. The molecular beacon sequences were labelled at the 3' end with DABCYL (the quencher) and at the 5' end with 6-FAM (the fluorescent label).

Annex to the application documents - subsequently filed sequences listing

[0044]

5

SEQUENCE LISTING

10

15

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<110> Amsterdam Support Diagnostics B.V.

<120> Testing endosymbiont cellular organelles and compounds
identifiable therewith

<130> P53526EP00

<140> 00204322.2

<141> 2000-12-04

<160> 31

<170> PatentIn Ver. 2.1

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EP 1 211 323 A1

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EP 1 211 323 A1

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EP 1 211 323 A1

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Claims

1. A method for determining functioning of a cellular organism comprising determining the relative ratio of a first endosymbiont cellular organelle nucleic acid and/or gene product thereof in a sample obtained from said organism in relation to the amount of a second nucleic acid and/or gene product thereof.
2. A method according to claim 1 whereby said relative ratio of a first endosymbiont cellular organelle nucleic acid and/or gene product thereof is determined in relation to the amount of a nuclear nucleic acid and/or gene product thereof detectable in said sample.
3. A method according to claim 2 wherein said nuclear nucleic acid comprises DNA.
4. A method according to claim 3 wherein said nuclear nucleic acid comprises DNA encoding a component of a small nuclear ribonucleoprotein or fragment thereof.
5. A method according to claim-2 wherein said nuclear nucleic acid comprises RNA.
6. A method according to claim 5 wherein said nuclear nucleic acid comprises RNA encoding a component of a small nuclear ribonucleoprotein or fragment thereof.
7. A method according to anyone of claims 1 to 7 wherein said endosymbiont cellular organelle nucleic acid and/or or gene product thereof comprises RNA.
8. A method according to claim 1 whereby said relative ratio of a first endosymbiont cellular organelle nucleic acid and/or gene product thereof is determined in relation to the amount of a second endosymbiont cellular organelle nucleic acid and/or or gene product thereof detectable in said sample acid.
9. A method according to claim 8 wherein said first nucleic acid comprises DNA.
10. A method according to claim 8 wherein said first nucleic acid comprises RNA.
11. A method according to anyone of claims 1, 2 or 8 wherein said first nucleic acid comprises DNA and wherein said second nucleic acid comprises RNA.
12. A method according to claim 11 whereby said second nucleic acid is in essence derivable by transcription from said first nucleic acid.
13. A method for determining therapeutic activity and/or possible side-effects of a candidate compound in determining its usefulness for treatment of malfunctioning of a cellular organism, comprising determining the relative ratio of an endosymbiont cellular organelle nucleic acid and/or or gene product thereof in a sample obtained from an organism
14. A method according to claim 13 wherein said organism or an essentially related organism has been provided with said compound.
15. A method for determining toxic activity of a candidate compound in determining its usefulness for causing malfunctioning of a cellular organism, comprising determining the relative ratio of an endosymbiont cellular organelle nucleic acid and/or or gene product thereof in a sample obtained from an organism
16. A method according to claim 15 wherein said organism or an essentially related organism has been provided with said compound.
17. A method for determining selective activity of a candidate compound against a first organism comprising determining therapeutic activity and/or possible side-effects of a candidate compound with a method according to claim 13 or 14 and/or determining toxic activity with a method according to claim 15 or 16.
18. A method according to claim 17 further comprising providing an essentially unrelated second organism with said compound.

19. A method according to claim 18 wherein said first organism comprises a pathogen and said second organisms comprises a host for said pathogen.
- 5 20. A method according to claim 18 wherein said first organism comprises a weed plant and said second organism comprises a crop plant.
21. A diagnostic kit comprising at least one means for performing a method according to anyone of claims 1 to 18
- 10 22. A kit according to claim 21, comprising at least one primer or probe selective for the amplification and detection of a nucleic acid related to or derived from endosymbiont cellular organelles.
23. A kit according to claim 22 wherein said primer or probe is listed in Table 1.
- 15 24. Use of a compound obtainable or selectable by a method according to the invention in the preparation of a medicament, a herbicide, an insecticide, an anti-parasiticum, an cytostatic agent or cytotoxic agent.
- 20 25. A medicament, a herbicide, an insecticide, an anti-parasiticum, an cytostatic agent or cytotoxic agent obtainable or selectable by a method according to the invention.
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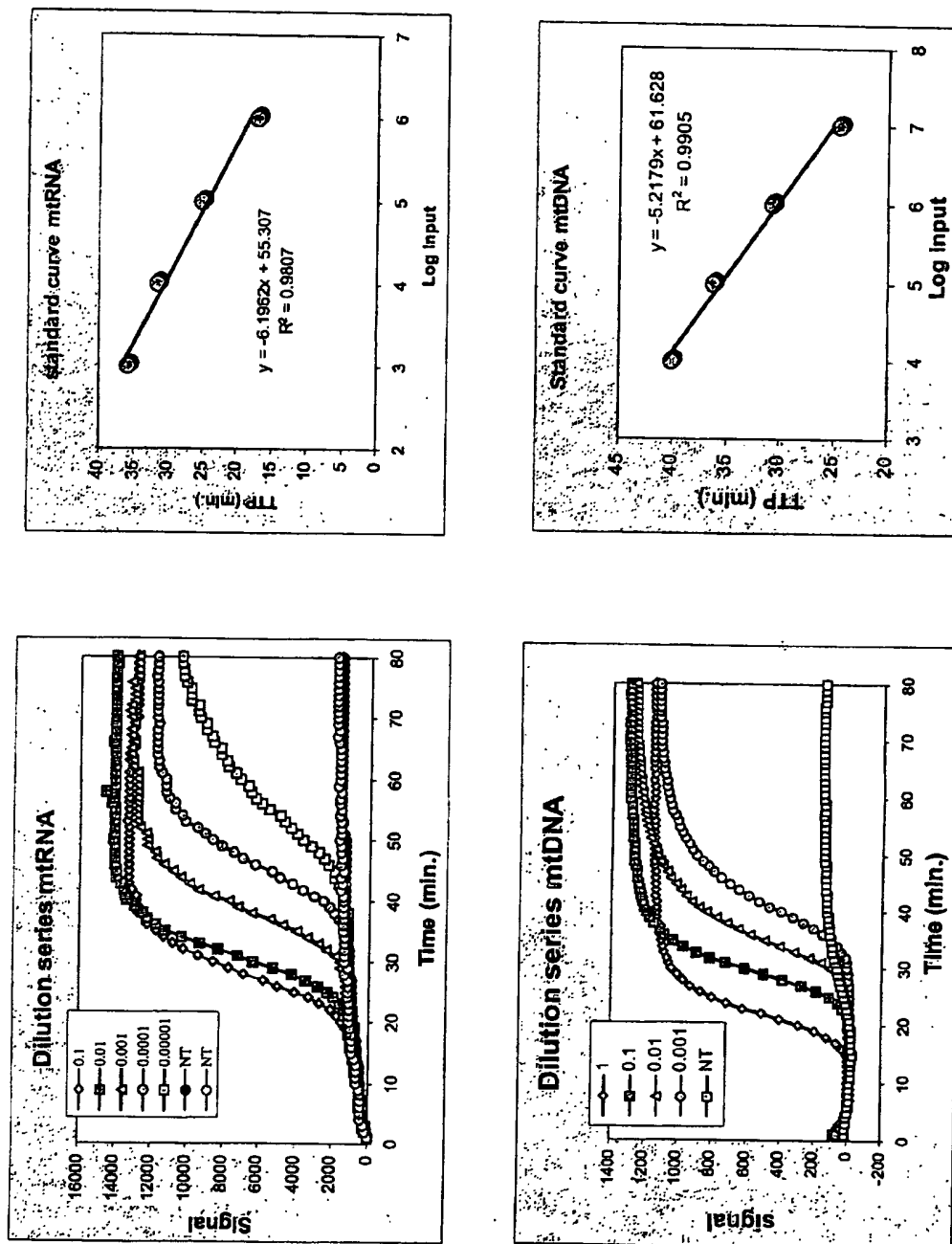


Figure 1

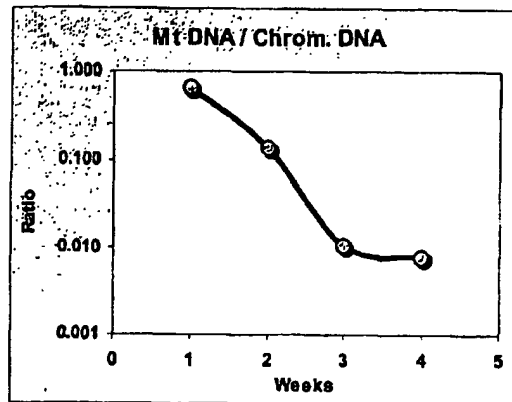


Figure 2

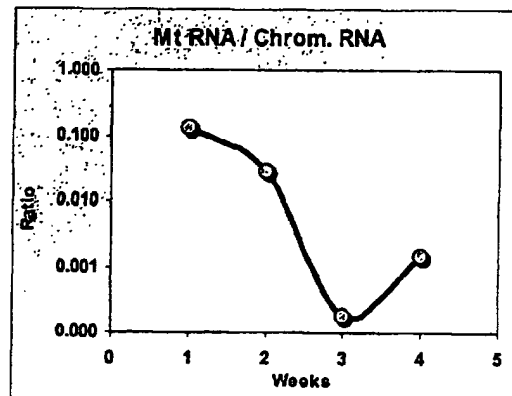


Figure 3

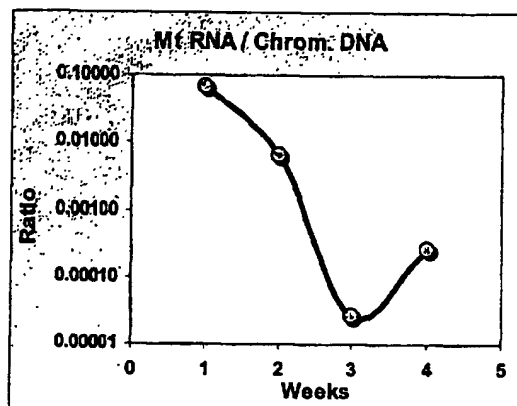


Figure 4

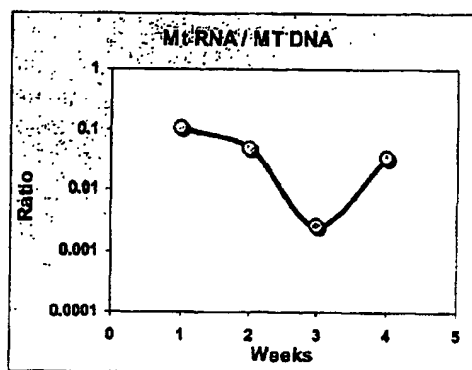


Figure 5

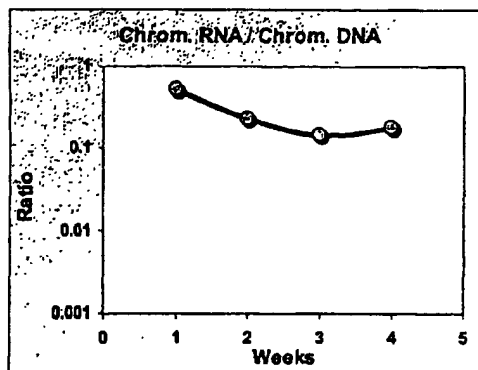


Figure 6

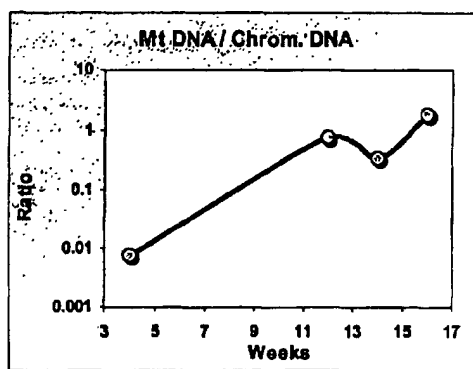


Figure 7

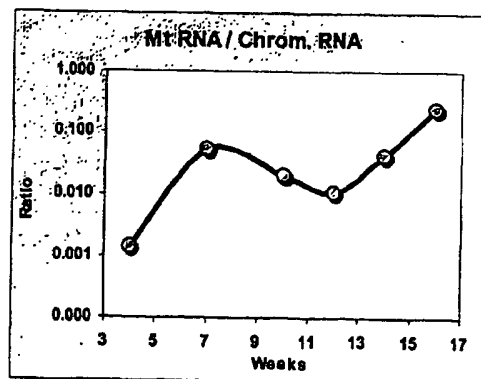


Figure 8

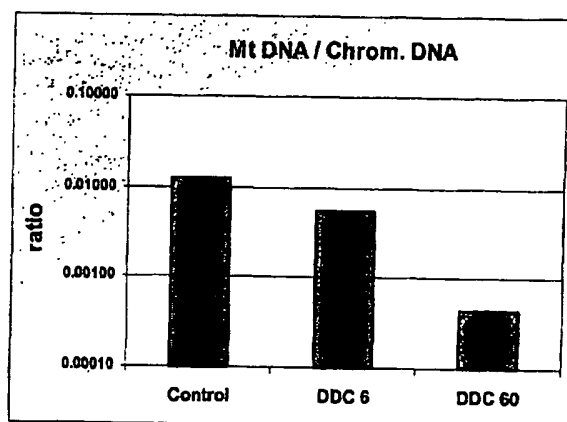


Figure 9

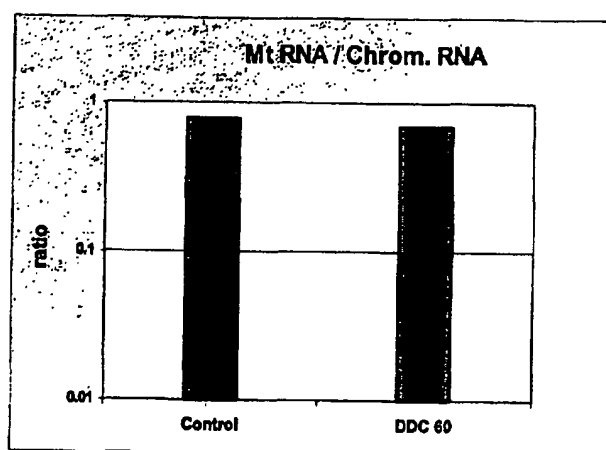


Figure 10

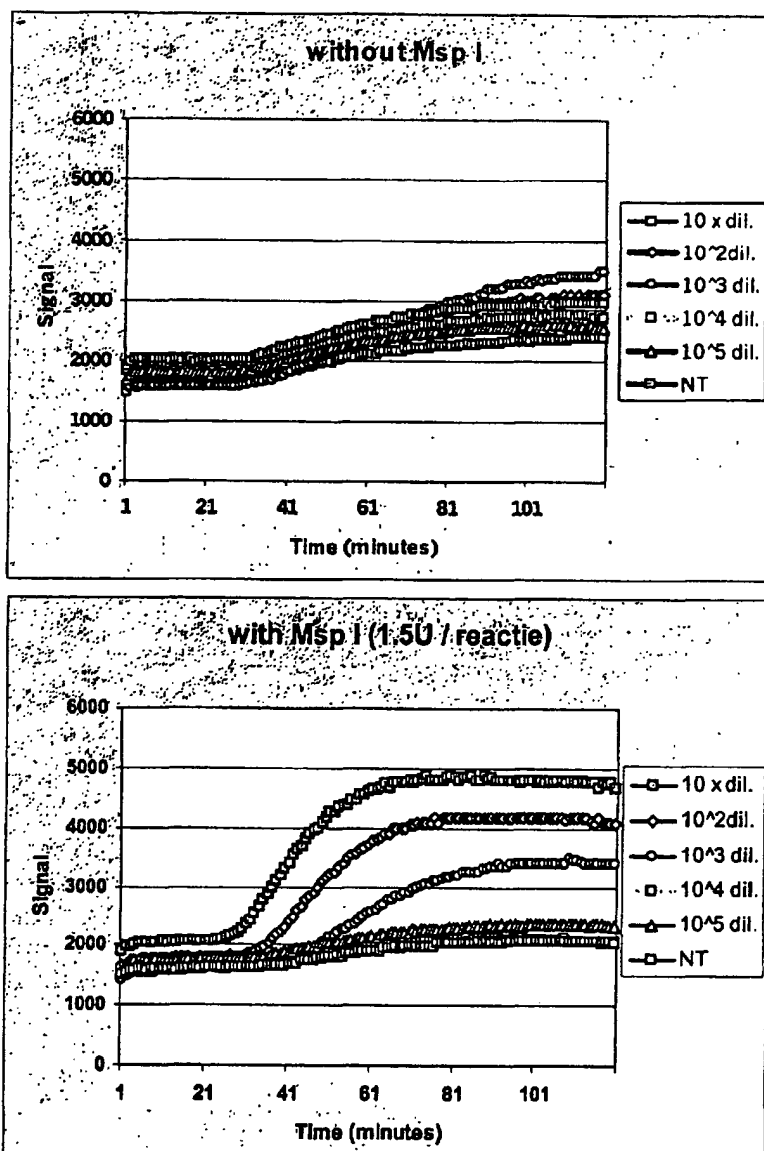


Figure 11. Comparison of Snrp DNA NASBA reactions with and without pre-treatment with restriction enzyme Msp I



European Patent
Office

PARTIAL EUROPEAN SEARCH REPORT

Application Number

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

EP 00 20 4322

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
X	WO 98 17826 A (MITOKOR) 30 April 1998 (1998-04-30) * page 13, line 6 - page 14, line 9; claims *	21,22	C12Q1/68
X	LEE H K ET AL: "Decreased mitochondrial DNA content in peripheral blood precedes the development of non-insulin-dependent diabetes mellitus." DIABETES RESEARCH AND CLINICAL PRACTICE, (1998 DEC) 42 (3) 161-7., XP000944595 abstract, discussion	1-4	
X	WO 99 66075 A (CLEVINGER WILLIAM ; FAHY EOIN D (US); MITOKOR (US); DAVIS ROBERT E) 23 December 1999 (1999-12-23) * claims *	1-4, 13-17	
			TECHNICAL FIELDS SEARCHED (Int.Cl.7)
			C12Q
INCOMPLETE SEARCH			
<p>The Search Division considers that the present application, or one or more of its claims, does/do not comply with the EPC to such an extent that a meaningful search into the state of the art cannot be carried out, or can only be carried out partially, for these claims.</p> <p>Claims searched completely :</p> <p>Claims searched incompletely :</p> <p>Claims not searched :</p> <p>Reason for the limitation of the search:</p> <p>see sheet C</p>			
Place of search		Date of completion of the search	Examiner
MUNICH		31 October 2001	Luzzatto, E
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>			

EPO FORM 1503 03.82 (P04C07)

European Patent
OfficeINCOMPLETE SEARCH
SHEET CApplication Number
EP 00 20 4322

Claim(s) searched completely:

23

Claim(s) searched incompletely:

1-22

Claim(s) not searched:

24,25

Reason for the limitation of the search:

1) Claims 24 and 25 relate to a compound obtainable by a method according to the invention, without providing any indication whatsoever as to any technical feature of these compounds which could allow a clear and unambiguous understanding of the scope of the claims. Claims 24 and 25 have thus not been searched.

2) Claim 21 relates to a kit comprising "at least one means" for performing a method as in claims 1-18, without however relating to any technical feature of the said "means", thus leaving the skilled person at a loss as to the scope of the claim. The claim has thus been searched only insofar as limited to the subject-matter of claim 22, i.e. to a kit comprising a primer or a probe selective for mitochondria. Claim 22 has been searched only insofar as related to a kit comprising a primer or probe selective for mitochondria (see also item 3 hereinbelow).

3) The description only provides examples relating to the determination of mitochondrial nucleic acid; examples 11 and 13 mention the use of primers and probes from *Oryza sativa* chloroplast DNA for DNA or RNA quantification, without however providing either any result or any experimental evidence as to a possible relationship between the result of the determination and the physiological status of the plant or the toxic/positive effect of a compound on the plant. Therefore, insofar as related to endosymbiotic organelles different from mitochondria, claims 1-20 lack support (Art. 84 EPC) to such an extent as to render a meaningful search impossible. The search has thus been carried out only insofar as related to mitochondria.

Application Number
EP 00 20 4322

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
X	ANDERSON C.M. ET AL.: "Mitochondrial electron transport complexes are decreased in skeletal muscle in type II diabetes mellitus" DIABETES, vol. 48, June 1999 (1999-06), page a259 XP000944142 US * abstract *	1,8	
X	WO 97 39149 A (COHEN SARIT ;LAVI SARA (IL); UNIV RAMOT (IL); KOHN KENNETH I (US)) 23 October 1997 (1997-10-23) * page 12, line 11 - page 14, line 11; claims 15-25 * * page 30, line 3 - line 19 *	1-4, 13-16	TECHNICAL FIELDS SEARCHED (Int.Cl.7)
X	BOULTWOOD J ET AL: "Amplification of mitochondrial DNA in acute myeloid leukaemia." BRITISH JOURNAL OF HAEMATOLOGY, (1996 NOV) 95 (2) 426-31., XP001029344 * abstract, materials and methods, discussion *	1-3	
X	TEPPER C G ET AL: "Resistance of mitochondrial DNA to degradation characterizes the apoptotic but not the necrotic mode of human leukemia cell death." JOURNAL OF CELLULAR BIOCHEMISTRY, (1993 JUL) 52 (3) 352-61., XP001029804 * abstract, discussion *	1-3	

-/-



European Patent
Office

PARTIAL EUROPEAN SEARCH REPORT

Application Number
EP 00 20 4322

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
X	ABBOTT A G ET AL: "QUANTITATIVE VARIATION IN COMPONENTS OF THE MAIZE MITOCHONDRIAL GENOME BETWEEN TISSUES AND BETWEEN PLANTS WITH DIFFERENT MALE-STERILE CYTOPLASMS." PLANT MOL BIOL, (1985) 4 (4), 233-240., XP001029181 * abstract, discussion *	1-3	
A	SU C.-Y.- ET AL.: "Selective reduction of creatine kinase subunit mRNAs in striated muscle of diabetic rats" AMERICAN JOURNAL OF PHYSIOLOGY, vol. 263, 1992, pages e310-e316, XP000943582 US abstract, "discussion" * the whole document *	1,2,5,8, 10	
A	WO 96 06191 A (AKZO NOBEL NV ;SILLEKENS PETER THEODORUS GERA (NL); TIMMERMANS EVE) 29 February 1996 (1996-02-29) * SEQ ID 5 * * page 5, line 14 - line 20; claims *	23	TECHNICAL FIELDS SEARCHED (Int.Cl.7)

EPO FORM 1503 03.82 (P04C10)

European Patent
Office**LACK OF UNITY OF INVENTION
SHEET B**

Application Number

EP 00 20 4322

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

1. Claims: 1-20 (completely), 22, 23 (both partly)

A method for determining the functioning of an organism comprising measuring the ratio of a first mitochondrial nucleic acid or protein relative to that of a second one, and a kit therefor, comprising the oligonucleotide of SEQ ID 1.

2. Claims: 22,23 (both partly)

A kit comprising SEQ ID 2

3. Claims: 22,23 (both partly)

A kit comprising SEQ ID 3

4. Claims: 22,23 (both partly)

A kit comprising SEQ ID 4

5. Claims: 22,23 (both partly)

A kit comprising SEQ ID 5

6. Claims: 22,23 (both partly)

A kit comprising SEQ ID 6

7. Claims: 22,23 (both partly)

A kit comprising SEQ ID 7

8. Claims: 22, 23 (both partly)

A kit comprising SEQ ID 8

9. Claims: 22, 23 (both partly)

A kit comprising SEQ ID 9



European Patent
Office

**LACK OF UNITY OF INVENTION
SHEET B**

Application Number
EP 00 20 4322

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

10. Claims: 22, 23 (both partly)
A kit comprising SEQ ID 10
11. Claims: 22, 23 (both partly)
A kit comprising SEQ ID 11
12. Claims: 22, 23 (both partly)
A kit comprising SEQ ID 12
13. Claims: 22, 23 (both partly)
A kit comprising SEQ ID 13
14. Claims: 22, 23 (both partly)
A kit comprising SEQ ID 14
15. Claims: 22, 23 (both partly)
A kit comprising SEQ ID 15
16. Claims: 22, 23 (both partly)
A kit comprising SEQ ID 16
17. Claims: 22, 23 (both partly)
A kit comprising SEQ ID 17
18. Claims: 22, 23 (both partly)
A kit comprising SEQ ID 18
19. Claims: 22, 23 (both partly)
A kit comprising SEQ ID 19
20. Claims: 22, 23 (both partly)



European Patent
Office

**LACK OF UNITY OF INVENTION
SHEET B**

Application Number
EP 00 20 4322

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

A kit comprising SEQ ID 20

21. Claims: 22, 23 (both partly)

A kit comprising SEQ ID 21

22. Claims: 22, 23 (both partly)

A kit comprising SEQ ID 22

23. Claims: 22, 23 (both partly)

A kit comprising SEQ ID 23

24. Claims: 22, 23 (both partly)

A kit comprising SEQ ID 24

25. Claims: 22, 23 (both partly)

A kit comprising SEQ ID 25

26. Claims: 22, 23 (both partly)

A kit comprising SEQ ID 26

27. Claims: 22, 23 (both partly)

A kit comprising SEQ ID 27

28. Claims: 22, 23 (both partly)

A kit comprising SEQ ID 28

29. Claims: 22, 23 (both partly)

A kit comprising SEQ ID 29

30. Claims: 22, 23 (both partly)



European Patent
Office

LACK OF UNITY OF INVENTION
SHEET B

Application Number
EP 00 20 4322

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

A kit comprising SEQ ID 30

31. Claims: 22, 23 (both partly)

A kit comprising SEQ ID 31

**ANNEX TO THE EUROPEAN SEARCH REPORT
ON EUROPEAN PATENT APPLICATION NO.**

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This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

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